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13. ABSTRACT (Maximum 200 Words)
Our laboratory has demonstrated the presence of different isoforms of proliferating cell nuclear antigen (PCNA) that display both acidic and basic isoelectric points (pI). Analysis of PCNA by two-dimensional polyacrlamide gel electrophoresis (2D PAGE) from both malignant and non-malignant breast cells and tissues established the exclusive presence of the acidic form of PCNA in malignant cells (which is now referred to as the cancer-specific form of PCNA or csPCNA). Additionally, a basic form of PCNA was also observed in the malignant cells, but this isoform was the only isoform found in non-malignant cells and tissues. Testing of numerous other malignant and non-malignant breast cells suggested that the csPCNA would be an excellent prognostic indictor of breast cancer. Further investigation confirmed that a 29 amino acid fragment derived from the PCNA binding domain of the XPG protein interacted with csPCNA and not the basic PCNA isoform. This led us to believe that the XPG peptide would be a specific and sensitive probe that would enable us to identify csPCNA in different tissue and serum samples. It is therefore our goal in this research to develop an enzyme linked immunosorbent assay (ELISA) that utilizes the 29 amino acid fragment of XPG to detect the presence of csPCNA in cells, tissues, and sera. Additionally, we plan on using the XPG peptide for immunocytochemical (IHC) staining allowing us to look for csPCNA in tissues of patients with breast cancer and uncertain malignant diagnosis. Following development of these assays, we will then screen our breast tissue and serum repositories, which we have been generating, and correlate our finding with other prognostic factors such as BRCA1 and 2, p53, $p27^{kip}$, and estrogen receptor status and thus validate the usefulness of csPCNA as an early diagnostic marker in breast cancer.

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INTRODUCTION

Our laboratory has demonstrated the presence of different isoforms of proliferating cell nuclear antigen (PCNA) that display both acidic and basic isoelectric points (pI). Analysis of PCNA by two-dimensional polyacrylamide gel electrophoresis (2D PAGE) from both malignant and non-malignant breast cells and tissues established the exclusive presence of the acidic form of PCNA in malignant cells (which is now referred to as the cancer-specific form of PCNA or csPCNA). Additionally, a basic form of PCNA was also observed in the malignant cells, but this isoform was the only isoform found in non-malignant cells and tissues. Testing of numerous other malignant and non-malignant breast cells suggested that the csPCNA would be an excellent prognostic indictor of breast cancer. Further investigation confirmed that a 29 amino acid fragment derived from the PCNA binding domain of the XPG protein interacted with csPCNA and not the basic PCNA isoform. This led us to believe that the XPG peptide would be a specific and sensitive probe that would enable us to identify csPCNA in different tissue and serum samples. It is therefore our goal in this research to develop an enzyme linked immunosorbent assay (ELISA) that utilizes the 29 amino acid fragment of XPG to detect the presence of csPCNA in cells, tissues, and sera. Additionally, we plan on using the XPG peptide for immunocytochemical (IHC) staining allowing us to look for csPCNA in tissues of patients with breast cancer and uncertain malignant diagnosis. Following development of these assays, we will then screen our breast tissue and serum repositories, which we have been generating, and correlate our finding with other prognostic factors such as BRCA1 and 2, p53, p27^{kip}, and estrogen receptor status and thus validate the usefulness of csPCNA as an early diagnostic marker in breast cancer.

Body
Aim 1: Development and validation of a quantitative sandwich enzyme linked immunosorbent assay (ELISA) capable of detecting the cancer specific from of PCNA (csPCNA) in malignant breast cell lines and breast tumor biopsy material.

We have repeated the GST pull-down experiments using the 29 amino acid (a.a.) XPG peptide several times and the results, as reported in the preliminary data, consistently shows that the GST-XPG fusion protein preferentially and specifically interacts with csPCNA and not the basic form of PCNA in MCF7 cells. These data confirm and strengthen the initial observation that the XPG peptide can be used as a selective tool to distinguish the presence of the cancer specific isoform of PCNA in malignant cells, tissues and sera.

Multiple attempts have been made to optimize the conditions using the XPG peptide to detect csPCNA from malignant breast cell lines and tissues using an ELISA format. Unfortunately, we have encountered numerous problems in developing this assay. The original ELISA assay presented in the preliminary data had a flaw that has been difficult to reconcile. Although this assay clearly showed a difference in the binding of PCNA from malignant and non-malignant cells, the ELISA assay did not adequately account for the differences in PCNA expression in the malignant cells compared to that of non-malignant cells. Therefore, the results could be due to the higher levels of PCNA in the malignant cells and not necessarily due to the specific identification of csPCNA. After numerous attempts, we were unable to repeat the result taking into account the PCNA levels in the cells. Because of this lack of repeatability, the original experimental conditions have been modified a several times.

First, we performed the assay in microcentrifuge tubes with antibody bound protein A beads rather than using a 96-well plate (figure 1). Protein A beads were first coated with polyclonal anti-PCNA antibodies.

MCF 7 cell extracts containing both csPCNA and the basic form of PCNA were incubated with biotinylated GST-XPG at 4°C by gentle rocking overnight in binding buffer (20mM Tris, 60mM NaCl, Potassium Phosphate
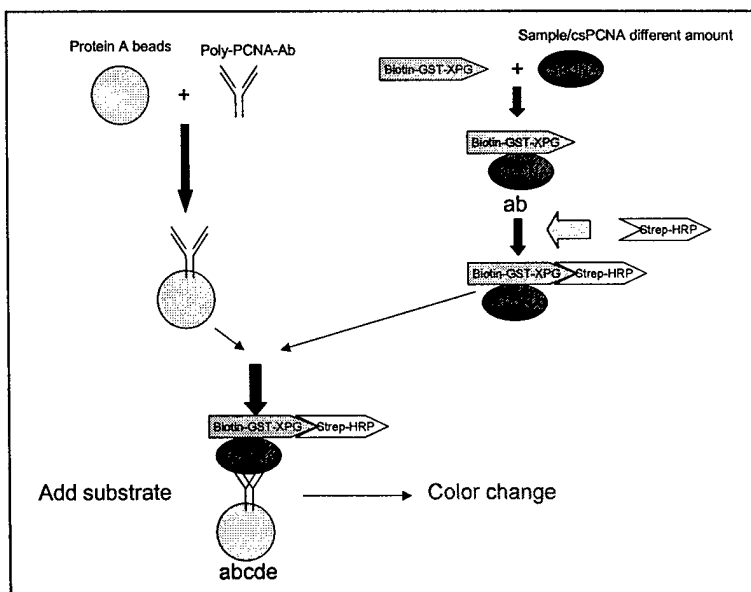

Figure 1. The protocol for the detection of csPCNA in microcentrifuge tubes. CsPCNA present in the samples was bound to biotinylated GST-XPG in solution, and streptavidin was subsequently added. Polyclonal PCNA antibodies pre-bound to protein A beads were then used to precipitate PCNA, and GST-XPG was detected.

Buffer, pH 7.4). It is essential to note that the binding buffer conditions are critical for the interaction between XPG and csPCNA. Horseradish peroxidase conjugated streptavidin (strep-HRP) was then added to the reaction mixture and incubated for one hour. Protein A beads coated with polyclonal anti-PCNA antibody was subsequently added and incubated for one hour. Protein A agarose beads were precipitated by centrifugation and unbound proteins were washed away. The HRP substrate, TMB, was then added, and the resulting blue color was measured by spectrophotometry.

Our results show that we are able to detect csPCNA by this method. However, a problem is encountered. When increasing concentrations of protein are added to the plate, a plateau or saturation of XPG signal is not achievable, but instead a decrease in signal is
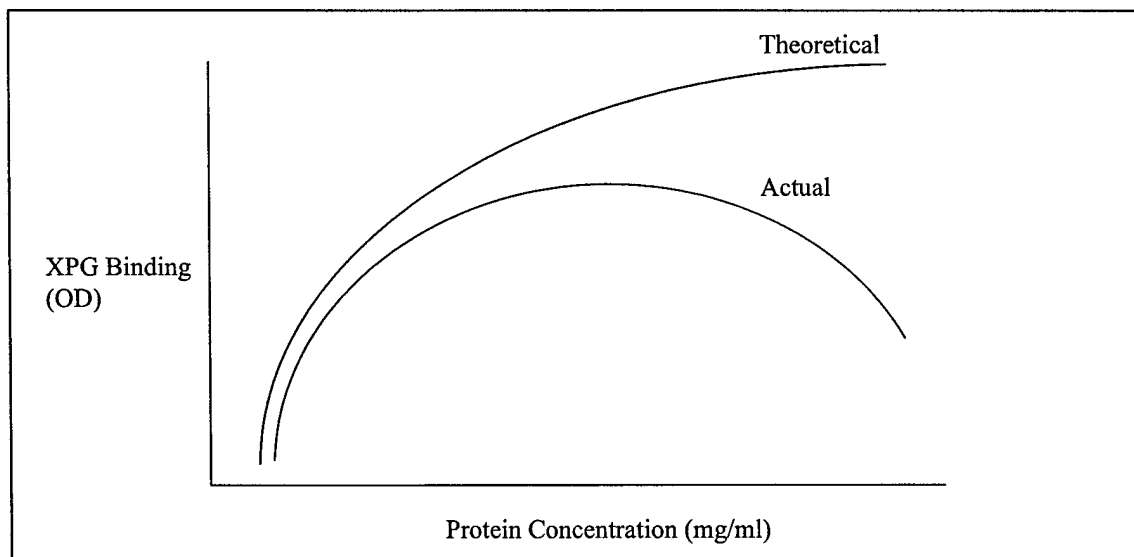

Figure 2. Detection of csPCNA using the microcentrifuge tube assay. The above graph illustrates a theoretical binding curve for csPCNA and a representative curve derived from our empirical data. Instead of the expected theoretical binding curve, our experimental data shows a sharp decrease in binding after a short plateau phase.

seen (figure 2). We believe this is due to the ability of PCNA to form multimers. For example, the first part of binding curve is indicative of csPCNA binding to the polyclonal antibody until the antibodies become saturated with PCNA (the plateau). After saturation of the PCNA antibody, PCNA can still interact with the beads through dimer and trimerization (i.e. 1:2 and 1:3 antibody to PCNA ratios). This leads to an apparent reduction in csPCNA binding because the PCNA/ PCNA interaction is significantly weaker than the PCNA / antibody interaction. Therefore, the loss of signal is due to loss of GST-XPG/ PCNA complexes in the washing steps, which were bound to other PCNA molecules and not to the anti-PCNA beads.

Next, we designed an alternative experimental method to detect csPCNA using the ELISA assay in a 96-well plate format. To do this a hybrid pull down/ ELISA was performed (figure 3). Initially, biotinylated GST-XPG was incubated with streptavidin conjugated agarose beads for 1 hour. After washing, MCF 7 cell extracts were added in binding buffer and incubated for 2 hours. Bound proteins (e.g. csPCNA [as shown with the GST-XPG pull-down assay]) were eluted of the streptavidin beads, and the amount of

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PCNA present in the eluate was detected using a PCNA ELISA. The PCNA ELISA utilizes a 96-well plate coated with polyclonal PCNA antibodies followed by detection with biotinylated monoclonal anti-PCNA antibodies pre-incubated with the sample. CsPCNA eluted from the XPG beads was subsequently bound to the biotinylated monoclonal antibodies in solution and the csPCNA/ antibody complexes were captured onto he surface of the ELISA plates by the polyclonal anti-PCNA antibodies. Detection of csPCNA/ monoclonal antibody was accomplished using HRP conjugated streptavidin; recent results show promise for detecting csPCNA using this method.

We postulated that the reason we did not get results with the assay method we originally proposed might be because of a weaker affinity of the XPG peptide for the csPCNA. Although the specificity of the XPG fragment for csPCNA is excellent, the sensitivity is not as good as we had anticipated, and we are therefore
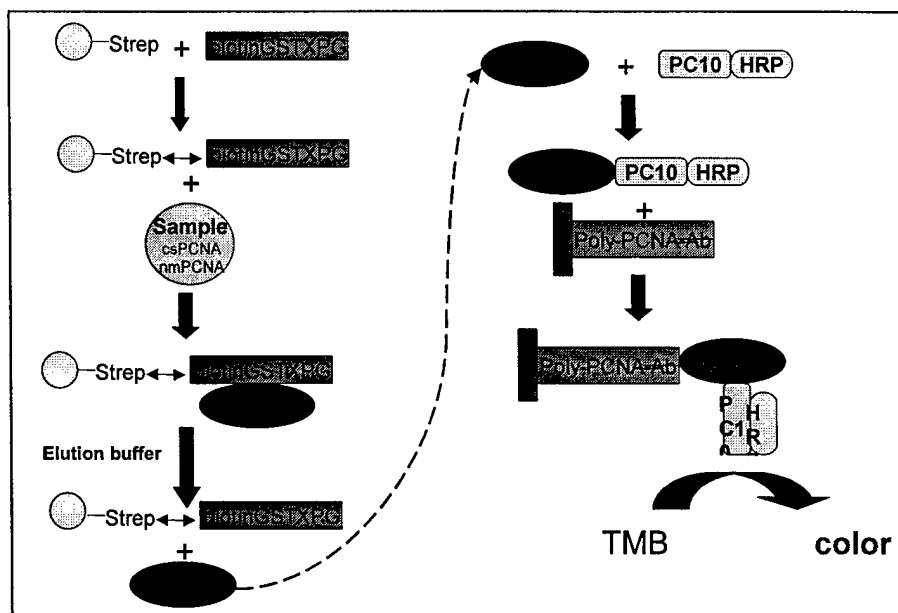

Figure 2. Procedure for the GST-XPG pull down/ ELISA hybrid experiment. Streptavidin conjugated agarose beads were pre-coated with biotinylated GST-XPG and used to precipitate csPCNA. CsPCNA was then incubated with biotinylated anti-PCNA antibodies before immobilization on the surface of the ELISA plate with a polyclonal anti-PCNA antibody. Bound csPCNA was then detected with streptavidin HRP.

actively pursuing alternative probes that have greater sensitivities. To do this we have begun to generate polyclonal antibodies directed to the region of PCNA that the XPG peptide interacts with. Our hopes are that the antibodies will give us a much higher sensitivity without losing the specificity of the XPG peptide. Additionally we are exhaustively searching for the post-translational modification of PCNA. This is being accomplished by purification of PCNA using various methodologies (GST-XPG pull down, p21 pull down, immunoprecipitation, in combination with hydrophobic interaction chromatography and size exclusion chromatography) in order to enrich and concentrate enough of the PCNA isoform to visualize by colloidal Coomassie staining of 2D PAGE. The spots visualized in the areas of the gel known to contain PCNA (by comparison to Western blots) were then removed, digested, and sequenced using an LCQAdvantage ion trap mass spectrometer. Briefly, the peptide digests are loaded onto a C4 sample trap cartridge present on a loading valve of a Surveyor AS300 autosampler. Trapped peptides are then eluted off the trap and onto a 0.15 X 15 cm Vydac Everest C18 capillary column and separated by a linear gradient of 5 to 60% acetonitrile in 0.25% formic acid. Eluted

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peptides are then ionized by microelectrospray and analyzed by triple play experiments. These data dependent experiments trap and fragment the eluting peptides by first isolating them, determining their charge state, and fragmenting them by collision-induced dissociation (CID). The fragment or MS/MS spectra can then be searched using different algorithms such as SEQUEST or MASCOT using databases of known proteins such as the NCBI's non-redundant database or the Swissprot. This data can also be used to identify post-translational modification, and the amino acid residues in which they reside. Although we have become quite efficient at this LC-MS/MS analysis technique, the identification of the PCNA modification has been confounded by visualization/resolution on 2D PAGE gels. First, a fairly purified sample is essential because it is extremely difficult to find one spot out of 100 let alone 1000, and, for reasons unknown, PCNA seems relatively resistant to staining with Coomassie blue. Due to these reasons, it has been very difficult to identify PCNA even after pull-down assays. Use of alternative stains and procedures for separating and visualizing the different isoforms of PCNA for LC-MS/MS is currently being undertaken.

Aim2: Development and validation of an immunocytochemistry assay using the PCNA-binding region of the XPG protein to selectively detect the csPCNA in breast tumor biopsies of uncertain malignant diagnosis.

Experiments using the XPG peptide to detect csPCNA by immunocytochemical staining have been performed on fresh frozen breast cancer tissues and normal breast tissues with. Briefly, we used 1:100, 1:500, and 1:1000 dilutions of our biotinylated GST-XPG to detect csPCNA in the tissue sections. Results suggest that we got very weak staining with the 1:500 and 1:1000 dilutions, while the staining in 1:100 group was too strong. Testing of concentration between 1:100 and 1:500 are now being performed and further optimization of the binding conditions is ongoing.

Additional assay development: Surface Enhance Laser Desorbtion/ Ionization time of flight mass spectrometry (SELDI-ToF MS) as a tool for the identification of csPCNA.

We have begun to test the utility of a specialized type of mass spectrometer, a SELDI ToF, for the identification of csPCNA. SELDI is a specialized type of matrix assisted laser desorbtion/ ionization (MALDI) mass spectrometer that used different chemistries on the surface of the sample plates to separate different molecules prior to analysis. One such plate or chip allows for the covalent attachment of a protein to the surface of the plate, and using this chip we have been able to immobilize the GST-XPG. We have then used the immobilized GST-XPG to "fish" csPCNA out of malignant cell extracts. Although the work is still very preliminary, the assay has consistently shown the presence of a peak with a molecular weight in the 29,000 MW range (PCNA MW=28,768). The drawback of this assay is that identification of this peak is not possible. Unfortunately, the SELDI mass spectrometer does not have an adequate mass accuracy and therefore the size of the peak changes sample to sample making accurate assignment of peak difficult. Additionally, the resolution combined with the mass accuracy of the ToF analyzer is also not ample for peptide mass fingerprinting, which could identify the peak after proteolytic

digestion on the chips surface. Despite these shortcomings, we continue to use the SELDI as another possible approach for identifying the csPCNA.

Key Research Accomplishments

- Accumulated samples from breast cancer patients
  - >300 sera
  - ~100 matched malignant and non-malignant tissues
- Developed partial and are continuing to develop funding for a bank of normal breast tissue (~200 volunteers) and intraductal carcinomas (IDC)
- Actively recruiting patients
- Developed a working pull down/ ELISA hybrid assay

Reportable Outcomes

We have developed a bank of over 100 matched malignant and non-malignant tissues and over 300 sera from breast cancer patients. We have also developed and are continuing to develop funding for an IDC and normal tissue bank, and are actively recruiting volunteers.

Abstracts:

Liu, Y., Hoelz, D., Tomic, D., Liu, J.Y., Hickey, R., Malkas, L. Effects of Interaction of Xermoderma Pigmentosum G (XPG) Fragment and Proliferating Cell Nuclear Anigen (PCNA) on Breast Cancer Cell Growth. 93rd Annual Meeting of the American Association of Cancer Research. April 2002. San Francisco, CA.

Tomic, D., Liu, Y., Hickey, R., Hoelz, D., Bechtel, P., Schnaper, L., Malkas, L. Cancer Specific Proliferating Cell Nuclear Antigen as a Novel Diagnostic Marker for the Detection of Breast Cancer. 93rd Annual Meeting of the American Association of Cancer Research. April 2002. San Francisco, CA.

Articles:

Liu, Y., Tomic, D., Schnaper, L., Hickey, R., Malkas, L.H. (2002) Diagnostic and Therapeutic Potentials for the interaction of Xermoderma Pigmentosum G Related Peptide and an isoform of proliferating cell Nuclear Antigen in Breast Cancer Cells. Breast Cancer Research and Treatment &
76(1): 210

Conclusions:

In the research conducted so far, we have developed both a working pull down/ ELISA hybrid experiment, and have begun to develop a immunocytochemical staining procedures to detect csPCNA in malignant cells and tissues. In addition to optimizing the use of the PCNA binding domain of the XPG protein to detect csPCNA by ELISA and immunocytochemistry, we are also exploring alternative means of detecting csPCNA that will permit us to create an even more sensitive assay. Some of the ways we are doing this is by developing polyclonal antibodies to the site of PCNA that interacts with XPG, and actively sequencing the different PCNA isoforms in hopes of discovering the position and type of post-translational modification present on PCNA, and utilize this knowledge for the development of specific detection agents.

So What Section

The importance of these experiments is underscored by the need for an early diagnostic marker or markers for breast cancer. Currently mammography is the most common and widely used test to diagnose breast tumors in women, and, although mammography currently the diagnostic method of choice, an earlier and more comprehensive test is still needed. Therefore, using the research and development put forth in this grant, we plan on creating a better and more efficient way of detecting breast cancer in women earlier and more comprehensively. Earlier detection of breast cancer may then lead to better and more successfully treatments and lower mortality rates.

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Curriculum Vitae

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EDUCATION

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POST-DOCTORAL RESEARCH

Development of the cancer specific form of PCNA as a biomarker for breast cancer, $p21^{WAF1}$ signaling in human cancer, isolation and identification of modified forms of PCNA in cancer, set-up, operation, and maintenance of an LC-MS/MS system
Indiana Cancer Research Institute
Indianapolis, IN

AWARDS AND RECOGNITIONS

Department of Defense Post-doctoral Breast Cancer Fellowship DAMD17—02-1-0467. Total costs $150,000. Cancer Specific Proliferating Cell Nuclear Antigen as a Novel Diagnostic Marker for Breast Cancer.
   (2002-Present)
Department of Defense Pre-doctoral Breast Cancer Fellowship DAMD17-99-1-9273. Total costs $66,000. The Regulatory Interactions of p21 and PCNA in Human Breast Cancer.
   (1999-2002)
Invited participant in the Molecular Biology and Pathology of Neoplasia Workshop, organized by the American Association for Cancer Research Keystone, Co.
   (1998)
Graduate Research Assistantship, Department of Pharmacology and Experimental
   Therapeutics. (1997-2001),
Full athletic scholarship including room and board to Kent State University.
   (1990-1995)

Region 7 team member at Junior Olympic Nationals for gymnastics, second place team.
(1990)
Top 25 individual at Junior Olympic Nationals and invited member of Top 25 Training
Camp, Olympic Training Center, Colorado Springs, Co.
(1990)

CURRENT RESEARCH FUNDING SUPPORT

**Department of Defense Medical Research and Development Command Breast
Cancer Research Program (2002-Present)** Cancer Specific Proliferating Cell Nuclear
Antigen as a Novel Diagnostic Marker for Breast Cancer. Total costs $150,000.

PUBLICATIONS
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Tomic, D., Liu, Y., Hickey, R.J., **Hoelz, D.J.,** Bechtel, P.E., Schnaper, L., Malkas, L.H. (2002) Cancer Specific Proliferating Cell Nuclear Antigen as a Novel Diagnostic Marker for the Detection of Breast Cancer. 43: 1041.

Hoelz, D.J., Markey, S., Maynard, D., Kowalak, J., Schnaper, L., Hickey, R.J., Malkas, L.H. (2002) Differential Binding of p21$^{WAF1/CIP1/SDI1}$ to PCNA Isoforms Present in Malignant Cells. DNA Symposium, Buffalo, NY.

INVITED TALKS
Hoelz, D.J., Maynard, D., Kowalak, J., Markey, S. Schnaper, L., Hickey, R.J., Malkas, p21 and PCNA: Signaling between DNA replication and repair. Proceedings of the American Association for Cancer Research. 44: .

ARTICLE REVIEWS
Biochemistry, Journal of Cell Biology

INVENTIONS
Hoelz, D.J., Malkas, L.H., Hickey, R.J. (2001) The Isolation of Modified and Non-modified Forms of Proliferating Cell Nuclear Antigen (PCNA) for Diagnostic/ Drug Development. (UMAB disclosure, patent pending)

Hoelz, D.J., Malkas, L.H., Hickey, R.J. (2002) Identification of a novel PCNA isoform that interacts with p21^{WAF1} for the development of novel anticancer agents. (IU disclosure)

RESEARCH SKILLS
Molecular Biology
The skills I have developed include molecular biological techniques such as agarose gel electrophoresis, the polymerase chain reaction (PCR), enzymatic assays (ligation, de-phosphorylation, restriction digestion, that have enabled me to construct prokaryotic and eukaryotic protein expression constructs that has allowed for production and isolation of mammalian gene products in large quantities. Utilizing these skills I was also able to develop and carry out strategies to construct plasmid DNA templates used for enzymatic assays such as the SV40 DNA replication assay. These skills will also allow me to further clone and develop other mammalian gene expression constructs and create different DNA templates useful in alternative enzymatic assays.

Enzymatic Assays
The enzymatic assays of which I am able to perform are activity assays for the DNA polymerases α, δ, and ε, the polymerases responsible for the synthesis of new DNA during mammalian DNA replication. Briefly, the assays involve the incorporation of radiolabeled deoxynucleotides into DNA primed templates followed by isolation of the DNA template and quantitation of the incorporated radioactivity by liquid scintillation. In addition, I am also able to perform the SV40 DNA replication assay. The assay encompasses all three phases of DNA replication and requires the SV40 virus large T-antigen and a DNA template containing the SV40 origin of replication in addition to mammalian DNA replication proteins. I am also skilled at performing topoisomerase assays, and using these enzymatic assays I have effectively been able to study the proteins responsible for DNA replication in mammalian cells and tissues. Additionally, I have performed *in vitro* transcription/translation assays to produce ^{35}S-methionine labeled recombinant proteins.

Protein Chemistry
I am also proficient at analyzing protein structure and protein/protein interactions using a variety of techniques. I have extensive knowledge and experience with multiple types of chromatography (ion-exchange, hydrophobic interaction, chromatofocusing, affinity, and size exclusion) using low and medium pressure chromatography systems (FPLC). I have experience in the setup, operation, and maintenance of and FPLC system (Biologic, BIO-RAD). I am also skilled in SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). I have extensive expertise in both immobilized pH strip (IPG) and tube gel methods of isoelectric focusing. These techniques have allowed me to compare the 2D-PAGE patterns of proteins isolated from malignant cells to those from non-malignant cells. For analysis of

the 2D images I have experience using both Phoretix 2D and Phoretix Evolution software packages (Nonlinear Dynamics). I am also skilled at identification of proteins separated by chromatography, SDS-PAGE, and 2D-PAGE by proteolytic digestion of individual proteins followed by analysis by matrix assisted laser desorbtion/ionization time-of-flight mass spectrometry (MALDI-ToF) in addition to analysis of internal peptide sequence by the HPLC electrospray tandem mass spectrometry (LC-MS/MS). I am also skilled in the immune and co-immune precipitation of proteins and protein complexes. In addition to co-immune precipitations, I am also skilled at GST pull-down assays and Far-Western blotting for the analysis of protein/protein interactions and adept at detecting proteins by conventional Western blotting.

Mass Spectrometry

I am skilled in the set-up, operation and maintenance of an LCQ-series ion trap mass spectrometer. In brief, I have expertise in plumbing a high performance liquid chromatography system (HPLC) to deliver low flow rates (1-5 µl/min by flow splitting) the to a micro-electrospray source on an LCQ-Advantage ion trap mass spectrometer (ThermoFinnigan). I am skilled at using capillary peptide traps for the concentration and desalting of protein digests prior to reversed-phase HPLC (RP-HPLC) using capillary columns (0.32 and 0.15 mm diameters). After RP-HPLC separation of peptides, I am adept at the setup and operation of the ion trap mass spectrometer for the Data-dependent analysis of the protein digests using "triple-play" experiments. For protein identification, I am also skilled at using two different MS/MS search algorithms, SEQUEST and MASCOT, which search the experimentally obtained MS/MS data against *in silico* proteolytic digests of known protein or translated DNA sequences to obtain amino acid sequence and protein identities. I am also adept at using these algorithms to search for post-translational modifications in addition to manual interpretation of the data. I am also skilled at maintaining and cleaning the HPLC and mass spectrometer to keep it in top working conditions including disassembly and cleaning of the electrospray source, the ion optics, and the mass analyzer, followed by reassembly and proper re-calibration and tuning of the instrument for optimal performance.